BMP4 and Neuregulin regulate the direction of mouse neural crest cell differentiation

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Abstract. The neural crest is a transient embryonic tissue that initially generates neural crest stem cells, which then migrate throughout the body to give rise to a variety of mature tissues. It was proposed that the fate of neural crest cells is gradually determined via environmental cues from the surrounding tissues. In the present study, neural crest cells were isolated and identified from mouse embryos. Bone morphogenetic protein 4 (BMP4) and Neuregulin (NRG) were employed to induce the differentiation of neural crest cells. Treatment with BMP4 revealed neuron-associated differentiation; cells treated with NRG exhibited differentiation into the Schwann cell lineage, a type of glia. Soft agar clonogenic and neurosphere formation assays were conducted to investigate the effects of N-Myc (MYCN) overexpression in neural crest cells; the number of colonies and neurospheres notably increased after 14 days. These findings demonstrated that the direction of cell differentiation may be affected by altering the factors present in the surrounding environment. In addition, MYCN may serve a key role in regulating neural crest cell differentiation.

Introduction

Neural crest cells originate from the dorsal margin of the neural plate, and can differentiate into various types of cells and tissues (1,2). Numerous neural crest cells have been isolated and characterized from different organs and tissues (3-7). During embryonic development, neural crest cells arise from the trunk region of the neural crest, migrate ventrally and aggregate adjacently to the dorsal aorta to form the primary sympathetic chain (8). The determination of neural crest cell fate is regulated by environmental factors from the extracellular surroundings (9). In addition, neural crest cells differentiate into various cell lineages according to their position in the embryo, inducing the formation of different cell types, including neurons, melanocytes, glial cells of the peripheral nervous system, endoneurial fibroblasts and endocrine cells (10-12)

Environmental factors may determine the differentiation fate of neural crest cells *in vitro*; neural crest cells were reported to be induced by a combination of secreted signals (11,12). Bone morphogenetic proteins (BMPs) are a unique group of proteins encoded by the transforming growth factor-β superfamily of genes, and have been reported as key regulators of embryogenesis (13). In addition, BMPs were observed to regulate the establishment of the embryonic body plan, dorsal-ventral patterning and the differentiation of neural cells (14-17). Additionally, BMP signaling has been demonstrated to affect the development of dorsal neural tube cells and formation of neural crest cells during a critical period prior to neural tube closure (18). Neuregulins (NRGs) are members of the epidermal growth factor protein family; it has been reported that NRGs are primarily expressed and secreted by neurons, and act on the surrounding glial cells (19). NRGs were demonstrated to induce the growth and differentiation of glial, epithelial and muscle cells *in vitro* (20-22). It has been reported that $NRG^{-/-}$ embryos died during embryogenesis and displayed heart malformations (23). NRGs may affect the survival, proliferation, migration, differentiation and myelination potential of Schwann cells (24-29); developing Schwann cells originate from neural crest cells that migrated along developing nerve fibers (10,30-32). Collectively, these findings suggest that environmental factors serve a critical role in neural crest cell differentiation. The present study aimed to determine the mechanism underlying neural crest cell differentiation in response to treatment with BMP4 and NRGs.

Myc activity has been reported to be a critical factor for the development and maintenance of stem cell properties; Myc has been demonstrated to control stem cell functions, including proliferation, differentiation and survival (33). Neural crest cells are generated from neural crest stem cells; as a migratory and multipotent cell population, neural crest cells can give rise to a variety of cell lineages during vertebrate development (34). N-Myc (MYCN) expression was observed in ~25% of neuroblastoma cases (35). A neuroblastoma is a tumor

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of the peripheral sympathetic nervous system and MYCN overexpression has been proposed as a tumorigenic event in the development of this disease (36,37). Furthermore, MYCN expression may be associated with the self-renewal ability and tumorigenic potential of neuroblastoma cells (36,38). Therefore, another aim of the present study was to determine whether MYCN could regulate the self-renewal ability of neural crest cells, and how the interaction between BMP4 or NGR and MYCN affects the fate of neural crest differentiation.

Materials and methods

Experimental animals. In the present study, 3 male and 9 female C57BL/6J mice (weight, ~22 g; age, ~9 weeks) were employed. All mice were housed under specific pathogen-free conditions as previous described (39). The animal experiments were approved by the Institutional Animal Care and Use Committee of Southwest University.

Cell culture and in vitro differentiation assays. Pregnant female mice (8.5-9 days gestation) were sacrificed via exposure to CO_2. The embryos were removed and washed in PBS. A total of 10-12 neural tube sections were excised with a scalpel and planted in 6-well cell culture plates containing Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 medium (DMEM/F12; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) medium as previously described (32), and photographed at 2, 24 and 48 h with a Nikon TS100 inverted microscope (Nikon Corporation, Tokyo, Japan) at a magnification of x40 or x100. Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) was used for analysis. All experiments were conducted using neural crest cells and their descendants that had not been cultured for >12 passages. For agent-induced differentiation assays, neural crest cells were cultured with 50 ng/ml BMP4 or 130 ng/ml NRG (both R&D Systems, Inc., Minneapolis, MN, USA) for 10 days in 37˚C. Neural crest cells treated with 1 μl/ml DMSO (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) served as the negative control.

Immunofluorescence. The tenth passage neural crest cells treated with BMP4, NRG or DMSO were fixed in 4% paraformaldehyde at room temperature for 15 min, permeated with PBS with Tween-20 (0.3% Triton X-100) at room temperature for 5 min and blocked with 10% goat serum (Beyotime Institute of Biotechnology, Haimen, China) at room temperature for 1 h. The cells were then incubated with primary antibodies at 4˚C overnight. The primary antibodies were as follows: Rabbit anti-glial fibrillary acidic protein (GFAP; cat. no. ab7260; 1:200; Sigma-Aldrich; Merck KGaA), chicken anti-Nestin (1:1,000; cat. no. NB100-1604; Novus Biologicals, LLC, Littleton, CO, USA), rabbit anti-SRY-related HMG-box 10 (Sox10; 1:300; cat. no. ab155279; Abcam, Cambridge UK) and mouse anti-neuronal-specific class III β-tubulin (TuJ1; 1:300; cat. no. ab78078; Abcam). Following washing with PBS, cells were incubated with secondary antibodies at room temperature for 2 h. All secondary antibodies were purchased from Invitrogen (Thermo Fisher Scientific, Inc.) and used at 1:1,000 dilution. The secondary antibodies were as follows: Alexa Fluor® 488-conjugated goat anti-mouse (cat. no. A-11001), anti-rabbit (cat. no. A-11008) and anti-chicken (cat. no. A-11039), and Alexa Fluor 594-conjugated goat anti-rabbit (cat. no. A-11012) immunoglobulin G. Then, all cells were washed with PBS and counterstained with DAPI (Beyotime Institute of Biotechnology) at room temperature for 20 min to detect nuclei, and images were captured with a Nikon Eclipse TE2000-E fluorescence microscope (Nikon Corporation) at a magnification of x100 or x200. Image-Pro Plus 6.0 software was used for analysis.

Retroviral production and transfection. The pBabe-puro/MYCN plasmid (Youbio, Hunan, China) was used to overexpress mouse MYCN in neural crest cells (MYCN-overexpressing neural crest cells), as previously reported (40), and the empty pBabe-puro plasmid as the control. Retroviral production and transfection were conducted as described previously (41). One day after retroviral transfection, the cells were cultured at 37˚C in the presence of 2 μg/ml puromycin for 3 days for resistance-based selection.

Soft agar clonogenic and sphere formation assays. For soft agar colony assay, a total of 1,500 pBabe-puro/MYCN or empty pBabe-puro neural crest cells in suspension were mixed with 0.3% low melting point agar containing DMEM supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). Cells were planted onto 6-well plates (1,500 cells/well) with a solidified bottom layer (0.6% low melting point agar in the same growth medium) for 14 days at 37˚C. For sphere formation assay, the medium in the aforementioned wells was replaced with DMEM supplemented with 20 ng/ml epidermal growth factor and 10 ng/ml basic fibroblast growth factor (both Gibco; Thermo Fisher Scientific, Inc.). After the 14-day culture, the colonies or the spheres were examined and photographed using a Nikon TS100 inverted microscope at a magnification of x100.

Western blot analysis. MYCN-overexpressing neural crest cells treated with BMP4, NRG or DMSO were suspended in RIPA lysis buffer and the total protein concentration determined using the Enhanced BCA protein assay kit (both Beyotime Institute of Biotechnology). Following this, 50 μg/lane of protein were separated by SDS-PAGE on 10% gel, transferred to polyvinylidene difluoride membranes. Membranes were then blocked with 5% no fat milk at room temperature for 1 h and then incubated with anti-MYCN (cat. no. ab24193; 1:1,000; Abcam) or anti-α-tubulin (cat. no. SAB4500087; 1:1,000; Sigma-Aldrich; Merck KGaA). Horseradish peroxidase-conjugated goat anti-mouse (cat. no. 5220-0341) or anti-rabbit (cat. no. 5220-0336) secondary antibodies (both 1:20,000; Kirkegaard & Perry Laboratories; SeraCare Life Sciences, Inc., Milford, MA, USA) were used as secondary antibodies. Proteins were visualized with BeyoECL Plus (Beyotime Institute of Biotechnology).

Results

Cells migrating from the neural tube are neural crest cells, which are characterized by Sox10 and Nestin expression. Explant culture has been successfully used to identify the properties of neural crest cells originating from the stem

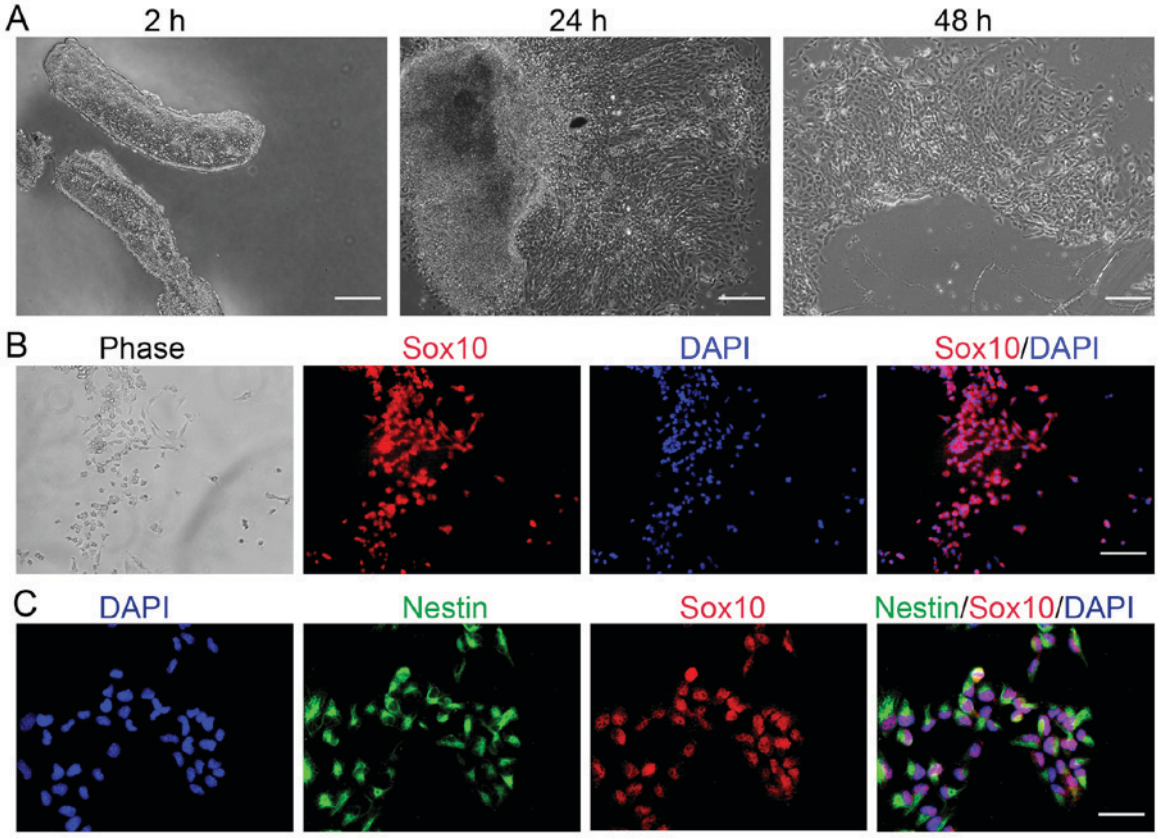

Figure 1. Cells migrating from the neural tube are neural crest cells, which are characterized by Sox10 and Nestin expression. (A) Cells migrated from the mouse embryo neural tube following culture for 2, 24 and 48 h. 2 h scale bar=250 μm; 24 and 48 h, scale bar=100 μm. (B) Phase images were taken and immunofluorescence analysis were conducted on cells that migrated from the trunk region of the neural tube. Scale bar=100 μm. (C) Immunofluorescence analysis of neural crest cells. Scale bar=50 μm. Sox10, SRY-related HMG-box 10.

cells of developing rodents (42,43). Therefore, explant culture was selected in the present study to determine whether any cells migrated from the neural tube of mice embryos. After a 2-h explant culture, the neural tube was clearly observed; after 24 h, neural crest cells were detected at the edges of the tissue blocks and some cells had migrated from the neural tube explants into the culture medium (Fig. 1A). The number of cells in the culture increased in what appeared to be a time-dependent manner, suggesting that the cells continued to migrate from the neural tube; after 2 days, more cells floated freely in the medium.

Sox10 is a unique HMG-box transcription factor expressed throughout the neural crest and in oligodendrocyte progenitor cells of the central nervous system (9,44,45). In the present study, cells were characterized via immunofluorescence to determine whether cells migrating from the neural tube expressed neural stem cell-associated markers. Compared with the phase image, immunofluorescent analysis revealed that all cells expressed Sox10 (Fig. 1B). Therefore, suggesting that cells migrating from the mouse embryo neural tube, which express Sox10, may be characterized as neural crest cells.

Nestin has been reported as a marker of neural stem or progenitor cells (46,47). Embryonic stem cell-derived neural precursor cells that had been further induced to differentiate into neurons may be selected based on the aforementioned strategy (48). The results of the present study demonstrated that neural crest cells were positive for Nestin (Fig. 1C), suggesting that neural crest cells may possess neural stem cell characteristics.

Neural crest cells maintain the potential of multilineage differentiation. The present study investigated the differentiation potential of neural crest cells in response to a variety of agents. A cell suspension was prepared from individual secondary colonies and plated onto glass coverslips. The results of the immunofluorescence analysis demonstrated that cells expressed the stem cell markers, Nestin and Sox10. In addition, neural crest cells were treated with BMP4 or NRG for 10 days. The majority of DMSO-treated cells exhibited a round and prominent nucleus, and abundant cytoplasm (Fig. 2). Cells treated with BMP4 exhibited neuronal cell morphology (49), with numerous long neuritic processes and small cell bodies that frequently formed aggregates (Fig. 2); however, cells treated with NRG exhibited Schwann-like cell morphology (50,51), with ovoid cell bodies, a prominent nucleus and natural bipolar extensions (Fig. 2). Therefore, these data suggested that BMP4 and NRG treatment may induce neural crest cell differentiation into neurons and Schwann cells.

To confirm the cell phenotype following treatment with BMP4 or NRG, immunofluorescence was conducted using anti-Nestin and anti-Sox10 antibodies. Compared with the untreated cell group, treatment with BMP4 or NRG resulted in a marked reduction in the number of cells expressing stem cell markers (Fig. 2), accompanied by a marked increase

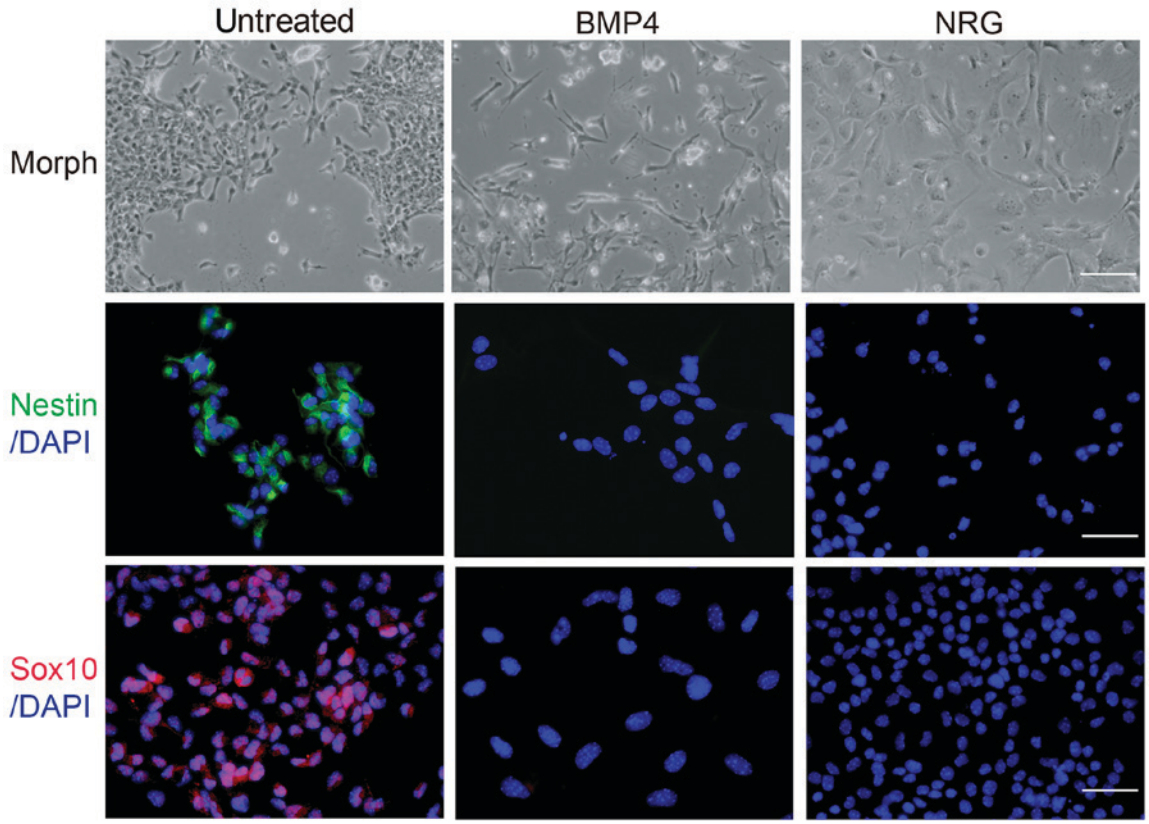

Figure 2. Morphology alters and stem cell marker expresion reduces in in neural crest cells following treatment with BMP4 and NRG. Morph, scale bar=100 μm; Nestin and Sox10 immunoflourescence, scale bar=50 μm. Sox10, SRY-related HMG-box 10; BMP4, bone morphogenetic protein 4; NRG, Neuregulin.

in the number of cells expressing differentiation markers (Fig. 3). BMP4-treated cells expressed the neuronal marker, Tuj1 (52), whereas NRG-treated cells expressed GFAP, which is a common marker of Schwann and glial cells (53), upon induction of differentiation. On the contrary, untreated cells expressed differentiation markers at markedly lower levels (Fig. 3). These differentiation analyses suggested that neural crest cells may possess the potential for multilineage differentiation and that environmental factors may control their fate.

Clonal sphere formation assay of MYCN-overexpressing neural crest cells suggests clonogenic self-renewal potential in vitro. MYCN upregulation was validated by western blotting (Fig. 4A). To investigate the function of MYCN in neural crest cells, the self-renewal and clonogenic abilities of MYCN-overexpressing neural crest cells were analyzed via soft agar and sphere formation assays. The result demonstrated that MYCN-overexpressing neural crest cells exhibited a higher number of colonies and larger colony sizes compared with the control group of neural crest cells (Fig. 4B). Similarly, the neurosphere formation ability of MYCN-overexpressing neural crest cells appeared to be enhanced compared with the neural crest cell control group under the same culture conditions. These results demonstrated that MYCN may promote the self-renewal ability of neural crest cells. The authors further investigated the effects of BMP4 and NRG on MYCN expression in MYCN-overexpressing neural crest cells; the protein expression levels of MYCN were markedly decreased in cells treated with BMP4 or NRG compared with the control cells (Fig. 4C).

Discussion

Neural crest cells have stem cell characteristics and have the ability to generate various types of cells and tissues during vertebrate development (54). In the present study, neural crest cells were isolated from mouse embryos and characterized by specific stem cell markers, including Sox10 and Nestin. Factors of the extracellular environment may affect the direction of neural crest cell differentiation. It has been reported that partitioning defective 3 homolog regulates the contact between neural crest cells and the timing of Schwann cell differentiation (55). In human neural crest stem cells, aligned electrospun fibers were revealed to promote differentiation towards the Schwann cell lineage (56). A recent study suggested that, during the neural differentiation of embryonic stem cells, miR-29b promoted the differentiation of embryonic stem cells into neural tube epithelial cells and inhibited their differentiation into neural crest cells (57).

It was previously reported that embryonic stem cells cultured with BMP4 differentiated into germ cells (58,59). BMP4 also regulated the proliferation and differentiation in epithelial and mesenchymal tissue compartments of the developing mouse ureter (60). In addition, BMP4 was reported to serve a key role in the differentiation of auditory neuron-like cells from bone-derived mesenchymal stromal cells (61). NRG is a type of polypeptide growth factor that serves a key role in

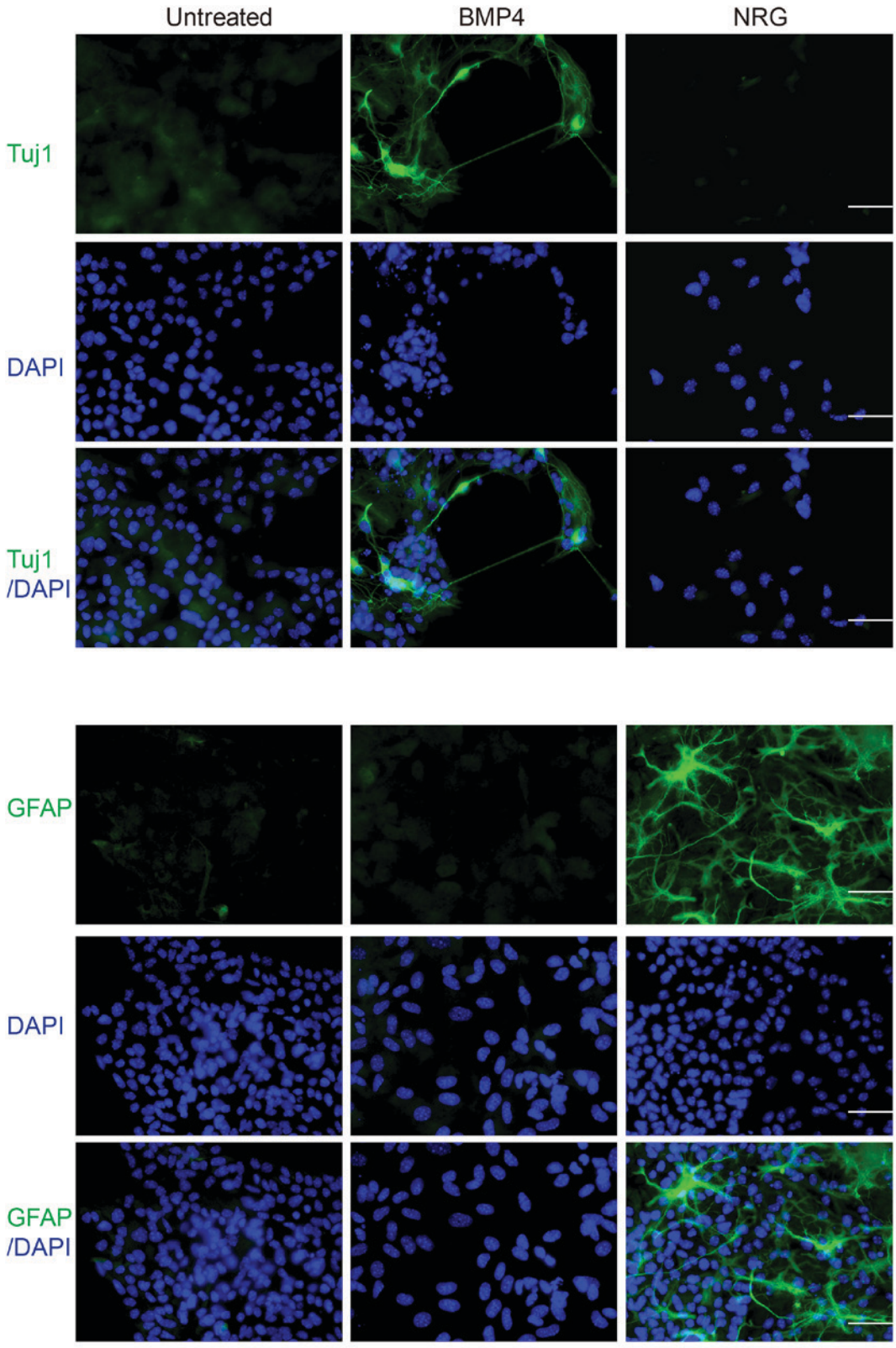

Figure 3. BMP4-treated and NRG-treated neural crest cells express differentiation markers. Immunofluorescence analysis of neural crest cells treated with BMP4 or NRG. The nuclei were visualized with DAPI staining. Scale bar=50 μm. BMP4, bone morphogenetic protein 4; NRG, Neuregulin; Tuj1, neuronal-specific class III β-tubulin; GFAP, glial fibrillary acidic protein.

the development and differentiation of the heart and nervous system (23,62). Generally, neural crest cells differentiate into glia, neurons and melanocytes in the mouse embryo (9,63). In the present study, the induction of differentiation via specific agents, BMP4 or NRG, revealed that neural crest cells may differentiate into neurons or Schwann cells, respectively, indicating that neural crest cells may alter their direction of differentiation according to their environments. Therefore,

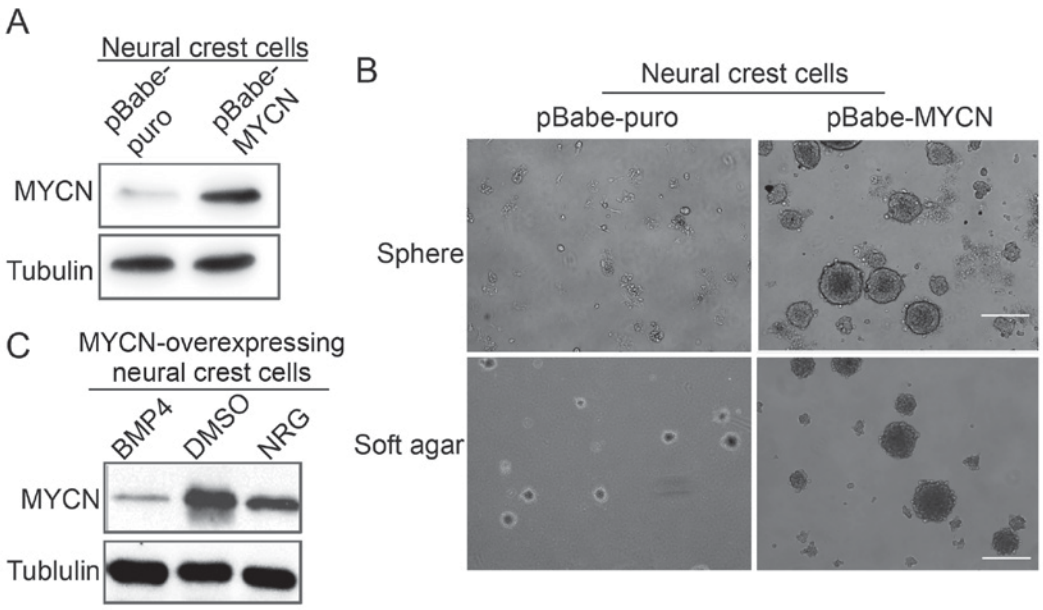

Figure 4. MYCN promotes neurosphere and soft agar colony formation, and BMP4 and NRG reduces MYCN expression. (A) Western blot MYCN expression in transfected neural crest cells. (B) Neurosphere formation and soft agar colonies in transfected neural crest cells after 14 days of culture. Scale bar=100 μm (C) MYCN protein expression following treament with BMP4 or NRG. MYCN, N-Myc; BMP4, bone morphogenetic protein 4; NRG, Neuregulin.

BMP4 and NRG could be considered as key factors in neural crest cell differentiation.

Self-renewal ability is an essential characteristic of stem cells, and enables the generation of daughter cells with the same developmental potential as their parental cells (64,65). Colony and sphere formation assays have been widely used to evaluate the self-renewal ability of individual stem cells (66-68). MYCN was reported as a key factor in the maintenance of embryonic stem cell-derived neural crest stem cells (69). The soft agar clonogenic and sphere formation assays revealed that MYCN-overexpressing neural crest cells were able to self-renew and generate progeny cells with the same self-renewal ability, MYCN-overexpressing neural crest cells developed more colonies compared with the neural crest cells transfected with empty vectors. Neuroblastoma is a cancer of neural crest stem cell lineage, many reports demonstrated that MYCN acted as an oncogene in neuroblastoma (35,36,70). MYCN served important roles in balancing proliferation, differentiation and cell death in neuroblastoma and normal neural crest cells (71,72). Therefore, it was hypothesized that MYCN-overexpressing neural crest cells acquire tumorigenic potential and that MYCN may regulate the development of neuroblastoma that originate from neural crest cells.

Of note, BMP4 could reduce MYCN expression and promote differentiation in neuroblastoma cells (73), and NRG was involved in neuroblastoma cell differentiation (74). These reports suggested BMP4 and NRG regulated neuroblastoma development. In the present study, BMP4 and NRG were shown to suppress MYCN expression in MYCN-overexpressing neural crest cells, which indicated that BMP4 and NRG may inhibit the maintenance of MYCN-induced stemness, suggesting there was cross-talk between MYCN and the BMP4 or NRG signaling pathway. Collectively, the findings of the current study indicated a molecular mechanism through which MYCN may promote the stemness of neural crest cells. Specific agents, including BMP4 and NRG, may decrease MYCN expression and induce neural crest cell differentiation. Therefore, the present study revealed that the direction of cell differentiation would be altered through modifying environmental factors. MYCN could serve a key role in regulating neural crest cell differentiation, and BMP4 and NRG may be regarded as novel inhibitors of MYCN amplification in neuroblastoma.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SZ performed the all the experiment with the exception of retroviral production and transfection, and wrote the manuscript. WL performed the statistical analyses. HFD and HC conducted the experiments. LY designed the current study and revised this manuscript. All the authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Animal Care and Use Committee of Southwest University (Chongqing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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